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Transcription of the phage-encoded Panton-Valentine leukocidin of *Staphylococcus aureus* is dependent on the phage life-cycle and on the host background

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ABSTRACT

Panton-Valentine Leukocidin (PVL) is a pore-forming, bi-component toxin secreted by *Staphylococcus aureus* strains epidemiologically associated with diseases such as necrotizing pneumonia and skin and soft-tissue infections. Here we demonstrate that transcription of the phage-encoded PVL (encoded in the *luk*-PV operon) is dependent on two major determinants: the phage life cycle and the host chromosomal background. Mitomycin C induction of PVL-encoding prophages from different community-acquired MRSA strains led to an increase in the amount of *luk*-PV mRNA as a result of read-through transcription from latent phage promoters and an increase in phage copy numbers. Failing prophage excision was reflected in a constant expression of *luk*-PV as in the case of strain USA300 suggesting that ΦSa2USA300 is a replication defective prophage. Additionally, we could show that *luk*-PV transcription is influenced by the *S. aureus* global virulence regulators *agr* and *sae*. We found a strong impact of the host background on prophage induction and replication when analyzing PVL-phages in different *S. aureus* strains. For example phage ΦSa2mw was greatly induced by mitomycin C in its native host MW2 and in strain Newman but to a considerably lesser extent in strains 8325-4, RN6390 and ISP479c. This discrepancy was not linked to the SOS response of the bacteria since *rec*A transcription did not vary between the strains. These results suggest a fine tuning between certain phages and their host with major impact on the expression of phage-encoded virulence genes.

INTRODUCTION

Staphylococcus aureus causes a variety of local and systemic infections in humans and is one of the most important community-acquired and nosocomial pathogens. The versatility of the organism is due its ability to produce a wide range of surface-exposed molecules which mediate interaction with the host cell as well as several secreted virulence factors. *S. aureus* necrotizing pneumonia and skin and soft tissue infections, which can also affect young, immunocompetent persons, is described as a threat associated with community-acquired (ca)MRSA and MSSA strains bearing the Panton-Valentine leukocidin (PVL) genes (Diederen & Kluytmans, 2006; Gillet *et al.*, 2002; Vandenesch *et al.*, 2003). The possible contribution of PVL to the virulence of *S. aureus* has been argued in studies using a variety of different animal models (Bubeck Wardenburg *et al.*, 2008; Diep & Otto, 2008; Labandeira-Rey *et al.*, 2007; Montgomery *et al.*, 2008; Voyich *et al.*, 2006; Wang *et al.*, 2007). PVL is a bi-component, pore-forming cytotoxin that targets host defense cells such as human polymorphonuclear neutrophils, monocytes, and macrophages (Genestier *et al.*, 2005; Kaneko & Kamio, 2004; Prevost *et al.*, 1995). The active form of PVL requires the assembly of two polypeptides, LukS-PV and LukF-PV, for which the corresponding genes (*lukS*-PV, *lukF*-PV) are carried by a prophage.

Although tightly linked to the phage genome and dependent on it for horizontal transfer, most of the phage-encoded virulence factors are integrated into the regulatory mechanism of the host. Examples of phage-related pathogenicity genes which are influenced by global *S. aureus* virulence regulators are plasminogen activator staphylokinase (encoded by *sak*), staphylococcal complement inhibitor (SCIN), chemotaxis inhibitory protein (CHIPS) (Rooijakkers *et al.*, 2006), and exfoliative toxin A (ETA) (Sheehan *et al.*, 1992). In a similar fashion, transcription of *luk*-PV was shown to depend on the activity of the regulators *agr*, *sar* and *sae*

(Bronner *et al.*, 2000; Voyich *et al.*, 2009). Additionally, the composition of the growth medium (Bronner *et al.*, 2000; Dumitrescu *et al.*, 2007) and subinhibitory concentrations of different antibiotics exerted a strong effect on PVL expression (Dumitrescu *et al.*, 2007; Stevens *et al.*, 2007).

Many prophages are induced by environmental conditions that lead to DNA damage, including exposure to exogenous agents such as antibiotics (Wagner & Waldor, 2002). We could show that phages are mobilized during chronic lung infection of patients with cystic fibrosis, possibly due to the strong selective pressure exercised on the pathogen (Goerke *et al.*, 2004; Goerke *et al.*, 2007). An enhanced expression of phage-encoded virulence genes after phage induction has been demonstrated for *S. aureus* (Goerke *et al.*, 2006a; Sumby & Waldor, 2003) and other bacteria (Wagner & Waldor, 2002). Here we show that transcription of *luk*-PV is greatly enhanced upon induction of PVL-encoding phages. This was due to an increase in phage copy numbers and to the activation of latent phage promoters after mitomycin C treatment of lysogens. Additionally, the bacterial host exerted a strong effect on *luk*-PV transcription and on prophage induction.

METHODS

Bacterial strains and lysogens. Bacterial strains used in this study are listed in table 1. Phage lysates were obtained from the appropriate strains by adding 1 µg/ml mitomycin C to cells in the exponential growth phase (OD_{600} = 0.8). After further incubation for 4h culture supernatants were sterilized using 0.45 pore diameter membrane filters (Millipore, Schwalbach, Germany). In order to obtain lysogens we spotted phages on the target strains and selected colonies growing in the center of a plaque. Single colonies were tested for phage integration by standard PCR using oligonucleotides specific for *luk*-PV (lukPV-for 5'-ACAGTGGTTTCAATCCTTCA-3', lukPV-rev 5'-CTTTTGGATTTGGCTTTGTA-3') and by pulsed-field gel electrophoresis fingerprinting. All investigations in this study were performed with at least two independently obtained lysogens. MW2 lacking the *hlb*-converting phage ΦSa3mw was obtained by screening mitomycin-treated bacteria on sheep blood agar plates for ß-hemolysin production. The respective colonies were picked and analyzed by PFGE for restoration of the intact *hlb* gene as described (Goerke *et al.*, 2006a) and for retention of the PVL phage. MW2*rec*A was obtained by transduction using a Φ11 lysate of strains ISP794*rec*A.

Mitomycin treatment. Strains were grown to the exponential growth phase (OD_{600} = 0.8, 2h) in CYPG medium (10 g/l casamino acid, 10 g/l yeast extract, 5 g/l NaCl, 20% glucose and 1.5 M phosphoglycerate) (Novick, 1991), at which point 300 ng/ml of mitomycin C or medium as control were added, followed by further incubation for 2h.

Quantitative real-time PCR for detection of extrachromosomal phage DNA. For DNA isolation bacterial cells were disrupted with glass beads (Sigma-Aldrich, München, Germany) in a high-speed homogenizer (Savant Instrument, Farmingdale, N.Y.) for 2 times 20s at 6500rpm. After heating for 2min at 100°C 1:100 dilutions of

the crude extracts were used. Quantification of the *att*P site of PVL-encoding phages and the *S. aureus* chromosomal gene *gyr* was performed by quantitative LightCycler PCR using the LightCycler SYBR Green Kit (Roche Biochemicals, Mannheim, Germany). Copies *att*P and *gyr* were determined using 10-fold serial dilutions of sequence-specific DNA standards as described (Goerke *et al.*, 2006a). Briefly, DNA standards were generated by regular PCR and amplicons were quantified spectrophotometrically. Master mixes were prepared following the manufacturer's instructions using the oligonucleotides specific for *att*P (MW2circle2-for 5'-AAAACAACCATTCGCATA-3' and MW2circle-rev 5'-ATTTTCCACAAGTGTTTGTC-3') and *gyr* (Goerke *et al.*, 2006a). At least two independent PCRs were performed for all samples. The specificity of the PCR reaction was verified by ethidium bromide staining on 3% agarose gels and by sequencing of the amplicons (4base lab, Reutlingen, Germany).

Northern analysis and quantitative RT-PCR. For Northern analysis *S. aureus* strains grown overnight in CYPG were diluted to an initial OD_{600} of 0.05 in fresh medium and grown with shaking (222 rpm) at 37°C to the early exponential phase (T1, OD_{600} = 0.8), late exponential phase (T2, OD_{600} = 1.9), and post-exponential phase (T3, OD_{600} = 8). Bacteria were harvested by centrifugation and dissolved in 1 ml Trizol reagent (Invitrogen, Karlsruhe, Germany). RNA was isolated from the bacterial pellet and Northern blot analysis was done as described previously (Goerke *et al.*, 2000). Specific primers for *agr* (RNAIII), *sae*, *hla*, *rec*A, *sak* (Goerke *et al.*, 2005; Goerke *et al.*, 2006a), and *luk*-PV (lukPV-for, lukPV-rev) were used to generate digoxigenin-labeled probes by PCR-labeling (Roche Biochemicals). Quantification of *luk*-PV transcripts was performed by Light Cycler RT-PCR as described (Goerke *et al.*, 2005) employing primer lukPV-for and lukPV-rev.

RESULTS

Transcription of *luk*-PV during growth

To investigate transcription of the *luk*-PV operon during growth we carried out Northern blot analysis of RNA from four MRSA strains harboring PVL-encoding phages. The strains selected represent typical caMRSA clonal lineages of *S. aureus*: the prototypic caMRSA MW2 and USA300 belong to MLST type ST1 and ST8, respectively. The two caMRSA from the collection of the German reference center for staphylococci at the RKI represent the European Clone ST80 (strain 02-02404) and the ST30 lineage (strain 03-1816). The *lukS*-PV and *lukF*-PV genes are transcribed as a single ~2.3 kb mRNA. In all four strains maximal expression was observed in the late exponential growth phase and transcription dropped post-exponentially with the exception of strain 03-1816, where mRNA levels remained equal (Fig. 1). The rapid decline in transcription in the stationary phase was unexpected for an *agr*-regulated gene (Bronner *et al.*, 2000). Therefore, we also examined the expression of the well-characterized *agr*-regulated toxin α-hemolysin (encoded by *hla*), for which the expected continuous increase into the late exponential phase was observed. Overall, strain MW2 produced considerably less *hla* than strains USA300, 02-02404 and 03-1816.

Correlation between transcription of *luk*-PV and phage induction

An increased transcription of phage-encoded staphylococcal enterotoxins and staphylokinase after induction of ß-hemolysin-converting phages that integrate into the *hlb* gene had been detected previously (Goerke *et al.*, 2006a; Sumby & Waldor, 2003). Activation of the SOS response by DNA-damaging agents led to the excision and replication of the phage, thereby increasing the amount of phage DNA template available for transcription. Similarly, expression of the phage-encoded *luk*-PV may

also be linked to the phage life cycle. Northern blot analysis revealed that mitomycin C treatment resulted in a marked increase in the amount of the ~2.3 kb *luk*-PV transcript in strains MW2 and 03-1816, but not in strain USA300 (Fig. 2A). Quantification of the *luk*-PV mRNA by qRT-PCR led to the detection 12 times more transcript in strain MW2 and 10 times more in strain 03-1816, whereas in USA300 the ratio remained equal. Mitomcyin C treatment also resulted in the production of a higher molecular weight mRNA in both strains with enhanced *luk*-PV transcription. By Northern analysis employing probes hybridizing to different parts 5' of *luk*-PV we could establish that the 4.9 kb transcript appearing in strain MW2 likely initiates from a region upstream of ORF MW1381 (data not shown). This is the putative holin gene of phage ΦSa2mw. Thus, the activation of latent phage promoters contributes to the increased expression of *luk*-PV.

RecA is the key enzyme of the bacterial SOS response. In all strains, subinhibitory concentrations of mitomycin led to increased *rec*A expression (Fig. 2A). It should be noted that the *rec*A transcription level did not differ between USA300 and the other strains and thus does not explain the differences in *luk*-PV transcription. In general, activated RecA leads to autocleavage of phage repressors and resumption of the lytic cycle. To prove that PVL-prophage induction and subsequent increased *luk*-PV transcription is *rec*A-dependent we included a *rec*A-defective derivative of strain MW2. No increase in *luk*-PV transcription after mitomycin treatment could be observed in this mutant, indicating recA dependent phage induction (Fig. 2 A).

So far, the results indicate differences in phage induction and replication in the four lysogens MW2, USA300, 03-1816 and MW2*rec*A. To test this, we established quantitative real-time PCR using specific oligonucleotides which span the *att*P sites of the excized PVL-encoding phages. In this PCR both the newly formed circular

phage genomes after rejoining of *att*P upon excision and all replicative forms of the phage can be detected. Quantification was performed with reference to the total amount of bacterial DNA represented by the chromosomal gene gyrase (encoded by *gyr*). Phage excision and replication was clearly detectable in strains MW2 and 03-1816 (Fig. 2B). Mitomycin treatment resulted in a 690-fold and a 1080-fold increase, respectively, in newly formed *att*P sites. In contrast, in strains USA300 and MW2*rec*A no phage excision was observed. The negative result of the latter suggests a missing cleavage of the prophage repressor by activated RecA. To exclude a potential PCR failure in the non-reactive lysogen USA300 due to extensive sequence variations in the *att* sites of the prophages, we analyzed the attL/R sites of ΦSa2USA300. A perfect match of the *att*P primers was found. Interestingly, replication of the *hlb*-converting phage of strain USA300, ΦSa3USA300, was detectable by a real-time PCR specific for the *att*P site of these phages (data not shown). Thus, the excision failure is a specific trait of ΦSa2USA300 and not due to a general dysfunctionality of the SOS response in strain USA300.

In summary, in strains MW2, 03-1816, and USA300 phage induction and *luk*-PV expression were correlated: in the first two strains phage replication is linked to an enhanced *luk*-PV transcription from internal phage promoters as well; in the latter the failing phage excision is reflected in a constant low expression of *luk*-PV. However, Western Blot analysis using specific antibodies against LukF-PV and LukS-PV revealed that elevated *luk*-PV transcription failed to result in increased amounts of protein (data not shown).

Phage induction is dependent on the bacterial host and is not influenced by co-infecting phages

To analyze the influence of the host chromosomal background on *luk*-PV transcription, we lysogenized the phage-cured strain 8325-4 with ΦSa2mw and Φ03-1816. With real-time *att*P PCR only a slight increase in phage particles could be detected for these lysogens after the addition of mitomycin (data not shown). Enhanced expression of *rec*A was detected by Northern blot analysis (Fig. 3A), but only a weak increase of *luk*-PV transcription occurred in the 8325-4 background. Thus, although a clear SOS response could be elicited by the mitomycin treatment in this host, both phages seem to be only weakly inducible, which is reflected in a low increase of *luk*-PV transcripts.

One reason for the differences in *luk*-PV transcription and phage induction between the original and the 8325-4 lysogens could be the presence of co-infecting phages aiding PVL-phage excision. Strain MW2 also carries the *hlb*-converting phage ΦSa3mw. Using a derivative of MW2 which was cured of ΦSa3mw we detected the same enhanced *luk*-PV transcription (Fig. 3B) and phage excision (data not shown) after mitomycin treatment that was found in the original double lysogen. Thus, co-infecting phages had no effect on PVL-phage induction and excision.

Transcription of *luk*-PV and phage induction is dependent on the chromosomal background

To further examine the impact of the bacterial background on PVL expression, the same phage, ΦSa2mw, was used to lysogenize different hosts: Newman, N315, s64c and the 8325-4 derivative RN6390. First we analyzed whether ΦSa2mw is inducible in the different host backgrounds. In the original host MW2 and strain Newman the highest increase (482-fold and 473-fold, respectively) in newly formed *att*P sites of

ΦSa2mw was detected after mitomycin treatment (Fig. 4A). In N315 the increase was less pronounced (46-fold) and only a very slight increase was observed in strains s64c and RN6390 (2-fold and 7.5-fold, respectively).

In the next experiments we concentrated on strains MW2 and the ΦSa2mw-lysogens of N315 and RN6390 as those differing most in phage induction. We included a ΦSa2mw lysogen of strain ISP479c which like RN6390 is a derivative of strain 8325-4 (Adhikari *et al.*, 2007). Mitomycin treatment resulted in an induction of the SOS system in all strains as demonstrated by the increased amount of *rec*A transcript detected in Northern Blot analysis (Fig. 4B). No differences in the level of *rec*A expression could be detected between the strains. When *luk*-PV transcription was analyzed, an increase in expression after mitomycin treatment was seen in strains MW2 and N315ΦSa2mw (Fig. 4B). An activation of latent phage promoters was also seen here. No response to mitomycin could be detected in the ΦSa2mw lysogens of strain RN6390 and ISP479c. Surprisingly, the latter lysogen, ISP479cΦSa2mw, showed a very high overall level of *luk*-PV expression independent of mitomycin induction. As a control the transcription of the staphylokinase was also determined in our strain collection since *sak* expression and phage replication are known to be linked (Goerke *et al.*, 2006a; Sumby & Waldor, 2003). All of the strains analyzed here harbor additional phages encoding this virulence factor (table 1). Mitomycin treatment resulted in an increase in *sak*-specific mRNAs in all strains chiefly due to the activation of latent phage promoters which can be deduced from the appearance of higher-molecular-weight bands in the Northern Blot (Fig. 4B). Additionally, replication of the respective phages could be proven by real-time PCR (data not shown). Thus, the enhanced expression of both phage-encoded virulence factors, *luk*-PV and *sak*, after activation of the SOS response by mitomycin C is dependent on phage replication. However, whereas the *sak*-

encoding, *hlb*-converting phages are induced in all strain backgrounds, the PVL-encoding phage ΦSa2mw is not.

Influence of regulatory loci on *luk*-PV transcription

Since the level of *luk*-PV transcription varied widely between strains, a strong influence of one or more host factors on PVL expression has to be assumed. Therefore, the activity of two global virulence regulators, *sae* and *agr,* was determined after mitomycin treatment. When RN6390 was compared with ISP479c the latter showed a higher *sae* transcription level, possibly explaining the discrepancies in *luk*-PV expression in these strains (Fig. 4B). It is consistent with this finding that the decrease in *sae* mRNA in RN6390 after mitomycin treatment was mirrored in a reduced *luk*-PV transcription. For further clarification we added a *sae*-replacement mutant of ISP479c to our investigation. The ΦSa2mw lysogen of ISP479c-*sae* displayed a dramatically diminished expression of *luk*-PV compared to the wild type. Thus, the virulence regulator *sae* positively regulates *luk*-PV transcription. No differences in *agr* expression between the strains could be observed - with the exception of strain N315, which proved to be an *agr* non-producer (Somerville *et al.*, 2003), explaining the very low level of *luk*-PV transcription. This was verified by analysis of an *agr*-replacement mutant of strain ISP479c, in which *luk*-PV mRNA was diminished compared to the *agr*-intact ISP479c lysogen, indicating that *agr* is a positive regulator of *luk*-PV albeit to a lower extent than *sae*.

Discussion

Prophage induction leads to an enhanced expression of *luk*-PV

S. aureus PVL is a pore-forming toxin secreted by strains epidemiologically associated with diseases such as necrotizing pneumonia and skin and soft-tissue infections (Gillet *et al.*, 2002). PVL-encoding phages are found in virtually all MRSA strains that cause community-associated infections (Chambers, 2005; Vandenesch *et al.*, 2003). Here we could show that transcription of PVL by *S. aureus* is dependent on two major determinants: the phage life cycle and the host chromosomal background. Mitomycin C induction of PVL-encoding phages led to an enhanced transcription of the virulence factor. The increase in *luk*-PV mRNA was a result of read-through transcription from latent upstream phage promoters and an increase in phage copy numbers due to phage replication. The new *luk*-PV transcript appearing after the addition of mitomycin was probably initiated from a latent promoter upstream of the ΦSa2mw holin gene. This corresponds well with earlier results obtained with *hlb*-converting phages showing that an increase in *sak* transcription was due to the read-through from upstream phage promoters (Goerke *et al.*, 2006a; Sumby & Waldor, 2003). In addition, phage replication was shown to play an essential role in the enhanced *sak* transcription (Sumby & Waldor, 2003). We could confirm the contribution of phage replication to *luk*-PV transcription by showing that the amount of *luk*-PV mRNA is directly linked to the quantity of extra-chromosomal phages: strongly replicating phages after mitomycin addition are strong PVL-producers (strain 03-1816), failing phage replication results in a constant *luk*-PV transcription (strain USA300). This link was emphasized when PVL-encoding phages were analyzed in different host backgrounds. The weak induction of phages ΦSa2mw and Φ03-1816 in strain 8325-4 was reflected in a small increase of *luk*-PV

mRNA. For many bacterial species it was shown that prophages are induced by environmental conditions and that induction is linked to enhanced production of phage-encoded virulence genes. In the case of *E. coli* numerous epidemiological studies have detected an association between increased severity of infection and treatment with antibiotics (Wagner & Waldor, 2002). For *S. aureus* it could also be demonstrated that there is a link between induction of prophages and subsequent virulence modulation of the bacterium by antibiotics which are often used during the treatment of infections (Goerke *et al.*, 2006a). A positive effect of beta-lactam antibiotics on phage replication was recently described (Maiques *et al.*, 2006). Subinhibitory concentrations of nafcillin (Stevens *et al.*, 2007) and oxacillin (Dumitrescu *et al.*, 2007) were found to enhance PVL expression, which is thought to contribute to worse outcomes of *S. aureus* infections after antibiotic therapy. The elevated PVL release observed in these studies might also be linked to an antibiotic-related induction of the prophage. However, in the current work the increase in *luk*-PV transcripts after mitomycin induction is not mirrored in a parallel increase in the PVL protein level. Sumby et al. were likewise unable to detect an increase in SEA production after mitomycin treatment, although ΦSa3ms prophage induction led to transcriptional up-regulation of *sea* (Sumby & Waldor, 2003). A posttranscriptional regulation seems to influence toxin production.

Prophage induction is dependent on RecA

Induction of PVL phages was directly dependent on the initiation of the SOS system. The prophage repressor cI is generally inactivated by the key enzyme RecA, leading to resumption of the lytic cycle. Lambdoid phage repressors are organized in two structural domains, the N-terminal domain responsible for contacts with the DNA and the C-terminal domain responsible for catalyzing the autoproteolysis reaction. A

linker region connects the two structural domains of the repressor. It contains a specific Ala (or Cys, Leu)-Gly cleavage site that is the target of C-terminal domain-catalyzed proteolysis (Koudelka *et al.*, 2004; Little *et al.*, 1980). Analysis of the cI-like repressor from ΦSa2mw revealed a structural organization dissimilar to that of the classical lambdoid repressors, making it difficult to envisage a RecA-dependent inactivation. However, we could show that in a *rec*A-negative MW2 derivative PVL-phage induction is completely abolished, thus providing evidence of a sensitivity of the phage repressor towards activated RecA. This suggests an alternative mode of cI inactivation in these PVL phages for instance by a RecA dependent activation of an anti-repressor as it was described for a mobile element of *Bacillus subtilis* (Bose *et al.*, 2008). Interestingly, other PVL carrying phages (e.g., ΦSa2985PVL and Φ108PVL) encode repressors which exhibit the typical lambdoid repressor structure (Ma *et al.*, 2008).

Influence of global virulence regulators on *luk*-PV transcription

Besides the phage life cycle, the *in vitro* growth conditions and the host chromosomal background exerted a strong effect on PVL production. Analysis of transcription during the growth of several caMRSA strains revealed a decline in the amount of *luk*-PV mRNA in the stationary phase, which is an uncommon expression pattern for a secreted, *agr*-regulated gene. Bronner et al. also observed a decline of expression post-exponentially when analyzing *S. aureus* strain V8 (Bronner *et al.*, 2000), whereas a strong transcription of *luk*-PV in the stationary phase up to 24h was shown by another group (Stevens *et al.*, 2007). One of our strains, 03-1816, also showed a prolonged expression of *luk*-PV into the post-exponential phase. Overall, the time course and the level of *luk*-PV expression seems to vary considerably between different *S. aureus* strains as was also shown by other groups (Hamilton *et*

al., 2007; Said-Salim *et al.*, 2005). Strains MW2 and USA300 in particular differed in the level of PVL expression. This was already observed by Montgomery et al. (Montgomery *et al.*, 2008), who could show that USA300 is more virulent than MW2 in a rat model of pneumonia. One reason for the observed strain-dependent PVL expression may be the activity of global virulence regulators. Several regulators like *agr, sar* and very recently *sae* were identified as controlling *luk*-PV expression (Bronner *et al.*, 2000; Voyich *et al.*, 2009). Here we could confirm that the virulence regulator *sae* positively influences transcription: in the *sae*-knockout mutant ISP479c-*sae* *luk*-PV specific mRNA was no longer detectable. This was also reflected in the prominent expression of *luk*-PV in strain ISP479c compared to RN6390. As shown here these two strains as well as the caMRSA strains USA300 and MW2 (Geiger *et al.*, 2008) vary clearly in *sae* expression emphasizing the strong positive effect of *sae* on *luk*-PV transcription.

Influence of the host background

The host chromosomal background also had a strong impact on phage replication. Mitomycin C treatment resulted in a pronounced increase of ΦSa2mw excision and replication in the native MW2 background and in strain Newman. However, when we analyzed ΦSa2mw induction in lysogens of strains 8325-4, RN6390, and ISP479c, we detected only a weak increase in phage copy numbers. This was not attributable to a failing initiation of the SOS system in these strains, since an obvious enhancement of *rec*A transcription was observed after mitomycin addition. Another possible explanation for the discrepancies in prophage induction in different strain backgrounds may be the presence of additional phages in the host that complement vital phage functions. Helper phages were shown to be important for the mobilization of staphylococcal pathogenicity islands (Lindsay *et al.*, 1998) and for the *E. coli*

phage P4 which requires a P2 helper phage for its assembly, packaging and lysis of the host cell (Christie & Calendar, 1990). In our case strain MW2 also carries phage ΦSa3mw, whereas strain 8325-4ΦSa2mw is a single lysogen. However, curing the second prophage of strain MW2 had no impact on phage replication or *luk*-PV transcription. Additionally, strains RN6390 and ISP479c also harbor phage Φ6390 (Goerke *et al.*, 2006b), which was clearly able to excise and replicate, as shown by the increased number of *sak* transcripts after mitomycin treatment. Phage ΦSa2mw was not inducible in the same background. Differences in the relative inducibility of phages were also noticed for lambdoid phages of *Escherichia coli* when spontaneous induction frequencies in double lysogens were compared (Livny & Friedman, 2004). In summary, there seems to be a strong link between the host background and the ability of a phage to resume the lytic cycle. How this interplay between a certain phage and its specific hosts functions remains to be elucidated.

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Table 1. Bacterial strains

Strain	Description[a]	Reference/Source
MW2 (USA400)	ST1 caMRSA, ΦSa2mw (*luk*-PV), ΦSa3mw (*sak*)	(Baba *et al.*, 2002)
MW2-ΦSa3mw	MW2 cured of ΦSa3mw	This work
ISP794*rec*A	ISP794, *rec*A::Tn*551*	(Fournier *et al.*, 2000)
MW2*rec*A	MW2, *rec*A::Tn*551*	This work
USA300	ST8 caMRSA, ΦSa2USA300 (*luk*-PV), ΦSa3USA300 (*sak*)	(Diep *et al.*, 2006)
02-02404	ST 80 caMRSA, uncharacterized Φ02-02404 (*luk*-PV) and ΦSa3-02-02404 (*sak*)	Robert Koch Institute, Wernigerode, Germany
03-1816	ST30 caMRSA, uncharacterized Φ03-1816 (*luk*-PV)	Robert Koch Institute, Wernigerode, Germany
8325-4	NCTC8325 phage-cured	(Novick, 1967)
8325-4ΦSa2mw	8325-4 lysogenized with ΦSa2mw	This work
8325-4Φ03-1816	8325-4 lysogenized with Φ03-1816	This work
Newman	Human clinical isolate, ΦNM1, ΦNM2, ΦNM3 (*sak*), ΦNM4	(Bae *et al.*, 2006; Duthie & Lorenz, 1952)
NewmanΦSa2mw	Newman lysogenized with ΦSa2mw	This work
s64c	clinical isolate	(Goerke *et al.*, 2006b)
s64cΦSa2mw	s64c lysogenized with ΦSa2mw	This work
RN6390	Derivative of 8325-4, Φ6390 (*sak*)	(Goerke *et al.*, 2006b;

		Peng *et al.*, 1988)
RN6390ΦSa2mw	RN6390 lysogenized with ΦSa2mw	This work
ISP479c	Derivative of 8325-4, Φ6390 (*sak*)	(Luchansky & Pattee, 1984)
ISP479cΦSa2mw	ISP479c lysogenized with ΦSa2mw	This work
ISP479c-*sae*	ISP479c, *sae*::kan, Φ6390 (*sak*)	(Geiger *et al.*, 2008)
ISP479c-*sae*ΦSa2mw	ISP479c-*sae* lysogenized with ΦSa2mw	This work
ISP479c-*agr* (ALC14)	ISP479c, *agr*::tetM, Φ6390 (*sak*)	(Wolz *et al.*, 2000)
ISP479c-*agr*ΦSa2mw	ISP479c-*agr* lysogenized with ΦSa2mw	This work
N315	HA-MRSA, ΦN315 (*sak*)	(Kuroda *et al.*, 2001)
N315ΦSa2mw	N315 lysogenized with ΦSa2mw	This work

a. only phage-encoded virulence factors important for this study are listed in parenthesis behind the appropriate prophage

Legends to the figures:

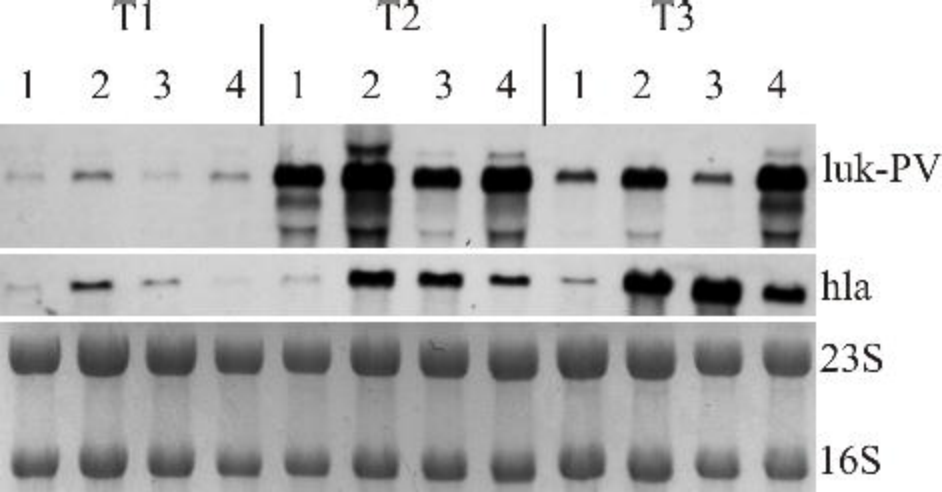
Figure 1. Northern blot analysis of RNA derived from strains MW2 (1), USA300 (2), 02-02404 (3) and 03-1816 (4) grown in CYPG to the early exponential phase (T1, OD_{600} = 0.8), late exponential phase (T2, OD_{600} = 1.9), and post-exponential phase (T3, OD_{600} = 8). The blots were hybridized using digoxigenin-labeled PCR fragments specific for luk-PV and hla. The ethidium-bromide stained gel is shown as a loading control.

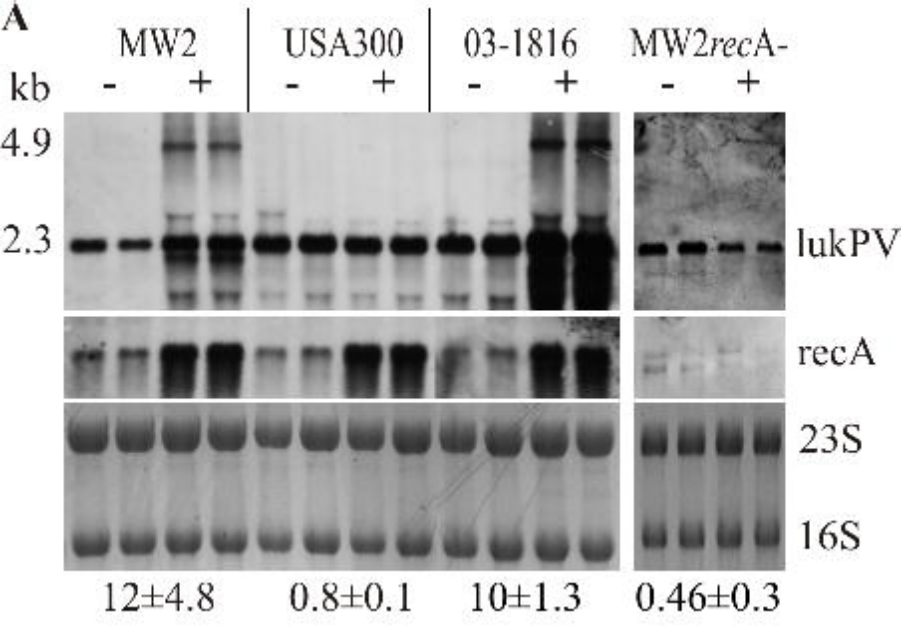
Figure 2. (A) Influence of mitomycin treatment on the transcription of *luk*-PV and *rec*A. Northern blot analysis was performed with RNA derived from strains MW2, USA300, 03-1816 and MW2*rec*A 2h after 300 ng/ml of mitomycin were added (+) or not added (-) to the culture. Each analysis was performed in duplicate. The ethidium-bromide stained gel is shown as a loading control. Below, the ratio of *luk*-PV mRNA derived from mitomycin-positive culture to that derived from negative cultures as determined by quantitative RT-PCR is given as fold-difference. Values from two independent RNA isolations each were used to calculate the mean ratio (± standard errors of the mean). (**B)** Influence of mitomycin treatment on PVL-phage induction in strains MW2, USA300, 03-1816 and MW2*rec*A. Phage induction was measured by a quantitative real-time PCR for the newly formed *att*P sites in reference to the total amount of bacterial DNA represented by the chromosomal gene *gyrase*, and the ratio of mitomycin-treated (300 ng/ml) to untreated cultures was calculated. Values from two independent PCRs each were used to calculate the mean ratio (± standard errors of the mean). nd, not detectable, copies *att*P below detection limit.

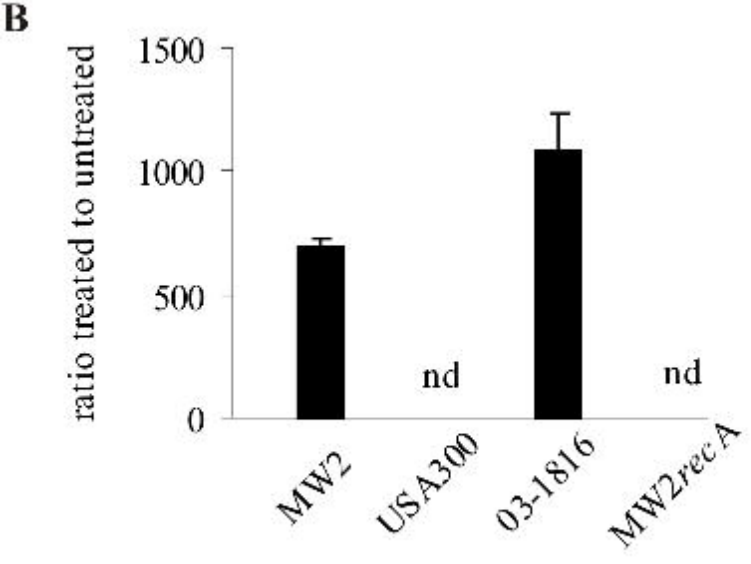
Figure 3. Influence of mitomycin treatment on the transcription of *luk*-PV and *rec*A in **(A)** ΦSa2mw and Φ03-1816 lysogens of strain 8325-4 and in **(B)** wild-type MW2 and MW2-cured of phage ΦSa3mw (MW2-ΦSa3mw). Northern blot analysis was performed with RNA derived from cultures 2h after 300 ng/ml mitomycin were added

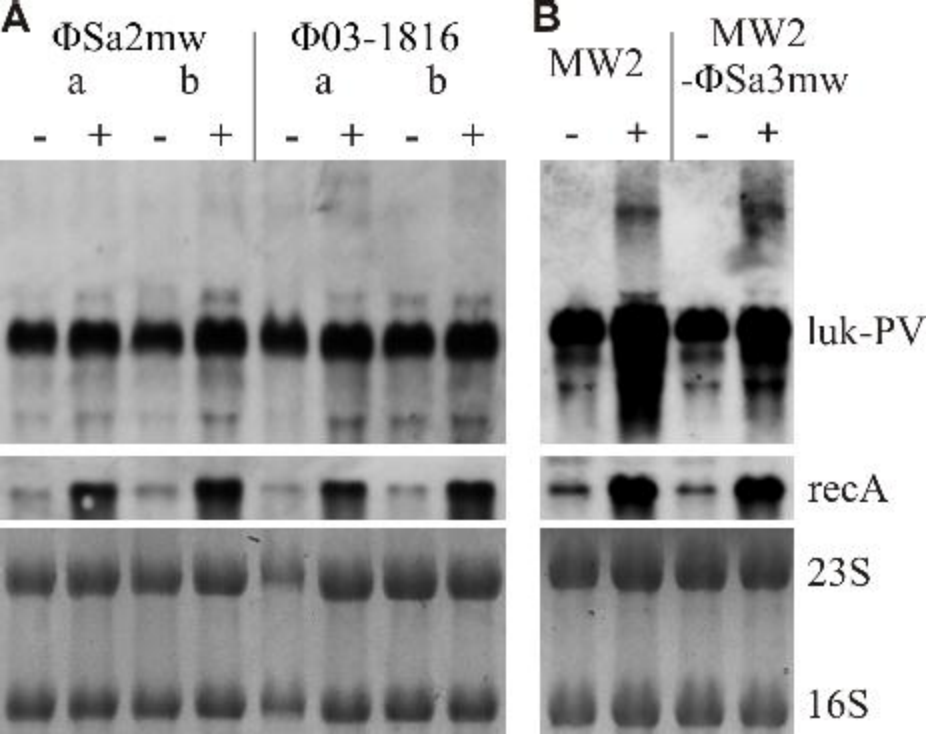
(+) or not added (-) to the culture. Where indicated, analysis was performed with two independently obtained lysogens (a, b). The ethidium-bromide stained gel is shown as a loading control.

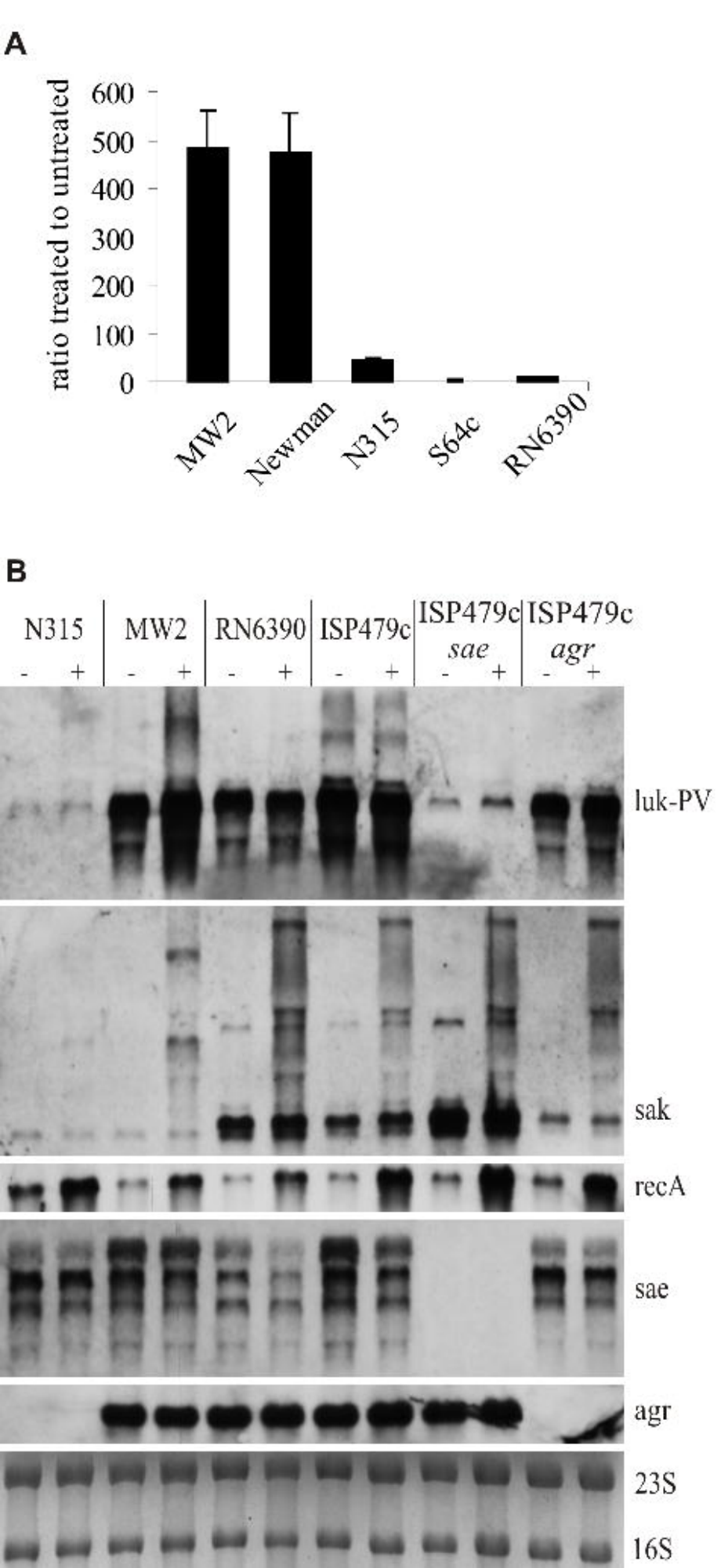
Figure 4. (A) Influence of mitomycin treatment on ΦSa2mw induction in different bacterial hosts: MW2, Newman, N315, s64c, and RN6390. Phage induction was measured by a quantitative real-time PCR for the newly formed *att*P sites in reference to the total amount of bacterial DNA represented by the chromosomal gene gyrase and the ratio of mitomycin-treated (300 ng/ml) to untreated cultures was calculated. Values from two independent PCRs each were used to calculate the mean ratios (± standard errors of the mean). **(B)** Influence of mitomycin treatment on the transcription of *luk*-PV, *sak*, *rec*A, *sae* and *agr* (RNAIII) in ΦSa2mw lysogens of strain N315, MW2, RN6390, ISP479c, ISP479c-*sae*, and ISP479c-*agr*. Northern blot analysis was performed with RNA derived from cultures 2h after 300 ng/ml mitomycin were added (+) or not added (-) to the culture. The ethidium-bromide stained gel is shown as a loading control. All results could be verified with independently obtained lysogens of each strain.


T1
T2
T3
1 2 3 4 1 2 3 4 1 2 3 4
luk-PV
hla
23S
16S


A
MW2
USA300
03-1816
MW2recA-
kb
-
+
-
+
-
+
-
+
4.9
2.3
lukPV
recA
23S
16S
12±4.8
0.8±0.1
10±1.3
0.46±0.3


B
ratio treated to untreated
1500
1000
500
0
nd
nd
MW2
USA300
03-1816
MW2recA


A
ΦSa2mw
a
b
Φ03-1816
a
b
- + - + - + - +
B
MW2
MW2
-ΦSa3mw
- + - +
luk-PV
recA
23S
16S


A
ratio treated to untreated
600
500
400
300
200
100
0
MW2
Newman
N315
S64c
RN6390
B
N315
MW2
RN6390
ISP479c
ISP479c
sae
ISP479c
agr
- + - + - + - + - + - +
luk-PV
sak
recA
sae
agr
23S
16S